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7 **Microvesicles released from *Giardia intestinalis* disturb host-**
8 **pathogen response in vitro.**
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ABSTRACT

Giardia intestinalis (G.I), is an anaerobic protozoan and the aetiological agent of giardiasis, a diarrhoea present worldwide and associated with poverty. G.I has a simple life cycle alternating between cyst and trophozoite. Cysts are transmitted orally to the stomach and transform to trophozoites in the intestine by a multifactorial process. Recently, microvesicles (MVs) have been found to be released from a wide range of eukaryotic cells. We have observed a release of MVs during the life cycle of G.I., identifying MVs from active trophozoites and from trophozoites differentiating to the cyst form. The aim of the current work was to investigate the role of MVs from G.I in the pathogenesis of giardiasis. MVs from log phase were able to increase the attachment of *G. intestinalis* trophozoites to Caco-2 cells. Moreover, MVs from *G. intestinalis* could be captured by human immature dendritic cells, resulting in increased activation and allostimulation of human dendritic cells. Lipid rafts participate in the MV biogenesis and in the attachment to Caco-2 cells. Nevertheless, proteomic analysis from MVs has shown no significant differences at the protein levels. An understanding of biogenesis and MV content of MVs derived from trophozoites might have important implications in the pathogenesis of the disease.

78 **Introduction:**

79

80 Cell-cell communication is mediated by secreted biomolecules, including peptides,
81 proteins, lipids and nucleic acids. These molecules are also present in extracellular
82 vesicles (EVs: mainly exosomes and microvesicles), which are released from different
83 cell types and are able to bind to receptors on target cells, triggering intracellular
84 signalling that modifies the physiological state of the target cells (Ratajczak et al.
85 2006).

86 EVs are found at elevated levels in cancer and in different acute and chronic
87 inflammatory diseases including sepsis, stroke, atherosclerosis and diabetes mellitus
88 (reviewed in Loyer X et al, 2014; Aurelian SM et al, 2014). They are also found in
89 physiological processes such as coagulation (Julich et al 2014).

90 Recently, many authors have described the involvement of EVs during the parasite-host
91 interaction. These authors have shown the presence of EVs of different sizes carrying
92 microRNAs, proteins and pro-inflammatory cytokines modulating the host cell (Marcilla
93 et al. 2014; Evans-Osses et al., 2015; Barteneva et al, 2013)

94 As the leading cause for protozoal diarrhoea worldwide, the intestinal parasite *Giardia*
95 *intestinalis* (Syn *G. duodenalis*, *G. lamblia*) is an important pathogen of humans and
96 animals causing morbidity and adversely affecting economies. *Giardia* has a peculiar
97 biology and represents an interesting biological model to understand evolution,
98 organelle function, and antigenic variation (Adam, 2001).

99 *Giardia intestinalis* has two evolutionary stages, the trophozoite, which is located in the
100 gut of animals and humans and which multiplies by binary fission, and the infectious
101 stage, the cyst, released into the environment in faeces.

102 *Giardia* belongs to the phylum Diplomonadida, unicellular eukaryotes that have
103 undergone considerable reductive evolution. The lateral gene transfer (LGT)
104 mechanism, an important evolutionary step in prokaryotes, has been shown in *Giardia*,
105 supporting this parasite to be included in an early branch of eukaryotic evolution
106 (Embley TM, Hirt RP, 1998). These findings provide insights into the evolution of
107 biochemical pathways in early eukaryote evolution, and could be important in
108 understanding the minimization, or even loss, of most cellular systems such as
109 mitochondria, peroxisomes, Golgi apparatus, and a classical endo-lysosomal system.

110 Pathological cysts are ingested via the oral route and symptoms usually occur after an
111 incubation period of 1–2 weeks., although half of *Giardia* infections are asymptomatic.
112 After emergence from cysts, the flagellated *G. lamblia* trophozoites colonize mainly the
113 upper small intestine. Trophozoites reside and replicate in the intestinal lumen and at
114 intestinal epithelial cells, but are not able to invade the mucosa. Although still poorly
115 understood, it is clear that the attachment of the parasite to the mucosal surface is the
116 critical point for its persistence in the host. The parasite contains a ventral disk that
117 seems to be important for attachment (Woessner and Dawson, 2012), while the flagella
118 contributes to correct positioning and orientation of the trophozoites before the
119 attachment. The parasite actively engages mucosal immunity and the infection
120 progresses with a low or absent inflammation in most cases (Oberhuber et al., 1997).
121 Most likely, mechanical effects, or some other, as yet not described mechanism,
122 produces villus and brush border microvillus atrophy, leading to digestive enzyme
123 deficiencies (Solaymani- Mohammadi and Singer, 2011), and chronic giardiasis can lead
124 to mucosal inflammation with pronounced villus loss (Hanevik et al., 2007); protease
125 activities may be a direct cause of diarrhoeas in giardiasis. Moreover, [Jimenez et al.](#)
126 [\(2004\)](#) found that excretory and secretory antigens (E/S Ags) from *G. lamblia* induced
127 an intestinal pathogenesis, which coincided with mucosal inflammation in BALB/c
128 mice. Oral administration of the E/S Ags not only stimulated production of antibodies
129 with parasitocidal activity, but also resulted in histological alterations within the
130 intestinal tissue that were comparable to those observed in natural and experimental
131 *Giardia* infections. After colonization the cyst formation represents a key step in the
132 life cycle of the parasite. This process involves cellular and molecular events . Lujan
133 H.D et al, 1996 reported that cholesterol starvation induces encystation.

134 Three encystation- specific cyst wall proteins (CWP1, 2 and
135 3) are expressed and concentrated in encystation-specific-
136 vesicles (ESVs) that circulate within the parasite before
137 being transported to the cyst wall (Lujan et al, 1996 ;
138 Reiner DS et al, 1990; Lauwaet T et al, 2007; Benchimol
139 and de souza, 2011). Synthesis of ESVs starts 4-6 hours
140 after encystation is induced and is completed with the
141 cyst formation by approximately 24 hours (Reiner DS et al,
142 1990) .. Interestingly, the protozoan could be able to release other kinds of vesicles

143 that could be speculated to be associated with the attachment to the intestinal cells and
144 pathogenesis. In preliminary work (Deolindo et al, 2013) we have shown that
145 *G.Intestinalis* may release MVs when exposed to different pHs and inducers. Now, We
146 have continued an in depth analysis of MV biogenesis and of the phenotype of the
147 extracellular vesicles released by the parasite.

148 We have hypothesized in this work that the response of *Giardia intestinalis* to
149 environmental stress conditions results in the active release of MVs from the plasma
150 membrane that modulate the host-parasite cell interaction.

151 **Materials and Methods**

152 **Cell culture**

153 A human colonic adenocarcinoma cell line, Caco-2 cell clone C2BBel [30], was
154 obtained from the American Type Culture Collection (CRL-2102). Caco-2 cells
155 (passages 57–72) were cultured at 37°C, 5% CO₂ in Dulbecco's Modified Eagle's
156 medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% foetal bovine serum
157 (FBS) (Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 mg/ml
158 streptomycin. Cells were fed every third day and passaged using 0.025% trypsin with
159 0.22 mM EDTA when 80–90% confluent.

160 **Parasite culture and *in vitro* encystation**

161 *Giardia lamblia* strain WB clone C6 was obtained from the American Type Culture
162 Collection (#50803). Parasites were grown in filter sterilized modified TYI-S-33
163 medium with 10% adult bovine serum and 0.05% bovine bile at 37°C in
164 microaerophilic conditions and subcultured when confluent. To collect parasites for
165 experiments, the medium was removed from the culture to eliminate unattached or dead
166 parasites. The tube was refilled with cold, sterile medium and trophozoites detached by
167 chilling on ice for 15 min. Parasites were collected by centrifugation (1500 x g for 5
168 min at 4 °C) and washed once with the plating medium of 90% complete DMEM / 10%
169 *Giardia* medium. Parasites were then counted using a hemocytometer, and diluted to the
170 appropriate number.

171 Encystation was induced as described previously (McCaffery and Gillin 1994). Briefly,
172 the pre-encysting cultures were grown to late log phase for 48 h in TYI-S-33 medium
173 (pH 7.1) without antibiotics. Encystation was initiated by removing the spent medium
174 and non-adherent cells and re-nourishing the adherent cells with an encystation medium

175 (TYI-S-33 medium adjusted to pH 7.8 and supplemented with 0.25 mg/ml bovine bile
176 and 5 mM lactic acid).

177 **Inhibition of Lipids Rafts** . Trophozoites from *G. intestinalis* stationary phase were
178 decanted, washed and resuspended in TYI-S-33 medium without FBS. The parasites
179 were incubated with 2.5, 5.0 and 10 μ M de M β CD for 1 hour at 37 C. After this time
180 the parasites were centrifuged at 1000 x g for 10 min and the pellet resuspended with
181 fresh TYI-S-33 medium and used in microvesiculation and adhesion essays.

182

183 **Adhesion assay**. The assay was carried out with stationary phase cultures of *G.*
184 *Intestinalis* trophozoites or with *G. intestinalis* trophozoites treated with M β CD (10
185 μ m). The parasites were decanted by chilling for 10 min in ice-cold PBS, at pH 7.2.
186 Trophozoite suspensions were centrifuged at 1000 x g for 10 min and resuspended to a
187 concentration of 1×10^6 / ml . Caco cells were seeded on a coverslip in a concentration
188 of 1×10^5 cells/ well. Suspensions of trophozoites were then co-incubated with cultured
189 cells in a 10 :1 ratio. Plates were incubated at 37°C in 10 % CO₂. After incubating for
190 1-3 h, unattached trophozoites were counted in a haemocytometer and the % adhered
191 cells determined . The effect of MVs were tested in the same experiments incubated
192 with different concentrations of purified MVs.

193 **Monocyte isolation**

194 Cells were obtained from leukocyte residues of healthy donors from the Blood and
195 Tissue Bank (Barcelona, Spain). Peripheral blood mononuclear cells (PBMCs) were
196 isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Sweden)
197 and T cells (CD3+) were depleted using the RosetteSep Human CD3 Depletion
198 Cocktail (StemCell Technologies, Seattle, USA). PBMCs depleted of T cells were
199 washed twice with washing buffer (400 x g, 5 min, RT) and were counted using
200 PerfectCount Microspheres (Cytognos, Salamanca, Spain).

201 Monocytes were then obtained by positive magnetic selection using the Easysep Human
202 anti-CD14 Positive Selection Kit (Stemcell Technologies, France) following the
203 manufacturer's instructions. Monocytes were >95% CD14+ and viable (as determined
204 by 7-AAD staining).

205 **Differentiation to iDCS**

206 Monocyte-derived dendritic cells (MDDCs) were generated by culturing monocytes
207 with the differentiation cytokines IL4 and GM-CSF for 6 days. Subsequently, isolated
208 monocytes were cultured at 1×10^6 cells/ml in complete medium composed of RPMI
209 1640 (PAA, Pasching, Austria) supplemented with 5% (v/v) heat-inactivated human
210 serum AB (BioWhittaker, Lonza), 2 mM L-glutamine (Sigma Aldrich, USA), 100 U/ml
211 penicillin (Cepa, Spain) and 100 U/ml streptomycin (Normon Laboratories, Spain), with
212 recombinant human IL-4 and GM-CSF (Miltenyi Biotech), both at a final concentration
213 of 1000 U/ml. After six days, immature dendritic cells (iDCs) were harvested by
214 collecting all media and incubating adhered cells with accutase (PAA, Pasching,
215 Austria) for 30 minutes at 37°C. iDCs were then washed with PBS (400 x g, 5 minutes),
216 counted and used for indicated experiments.

217 **Generation of MVs**

218 Plasma Membrane Vesicles (MVs) were produced from the *Giardia intestinalis*
219 trophozoite. Parasites were grown in TYI-S-33 medium with 10% adult bovine serum
220 and 0.05% bovine bile at 37°C in microaerophilic conditions and subcultured when
221 confluent. To collect parasites for experiments, the medium was removed from the
222 culture to eliminate unattached or dead parasites. The tube was refilled with cold, sterile
223 medium and trophozoites detached by chilling on ice for 15 min. Parasites were
224 collected by centrifugation (1500 x g for 5 min at 4 °C) and were then counted using a
225 hemocytometer, and diluted to 1×10^6 cells/ ml. The parasites were incubated in 1ml
226 serum-free culture medium (Yi-S) and stimulated with 1mM CaCl_2 for 1h at 37°C. This
227 way, MV production was stimulated so it prevailed over exosome release, and thus an
228 enriched MVs-containing medium was obtained. After incubation, all medium was
229 collected, centrifuged at 2,500 x g for 5 min and the supernatant was further centrifuged
230 twice at 4,000 x g for 30 min. Afterwards, supernatant was ultracentrifuged at 100,000 x
231 g for 1h 30 min and the pellets, containing concentrated MVs, were collected. MVs
232 were suspended in PBS and were approximately quantified according to their protein
233 content (Bradford assay). Finally, MVs suspensions were dried using speed vacuuming
234 for storage and shipping. Dried MVs were resuspended in PBS and kept at 4°C until
235 further use.

236

237

238

239 *Flow cytometry*

240 MVs were quantified by counting in a BD FACScaliburTM Flow Cytometer (Becton,
241 Dickinson and Company) using dot plots with SSC and FSC in log scale. Quantification
242 of MVs by FACS analysis was further validated by protein quantification with the
243 Bradford assay.

244 For surface phosphatidylserine detection MVs were resuspended in 200 µl of annexin-
245 binding buffer (ABB - HEPES 10 mM, NaCl 140 mM, CaCl₂ 2.5 mM, pH 7.2) and
246 incubated with 25 µg/ml AnnexinV-FITC (Sigma-Aldrich) for 30 min at room
247 temperature. MVs were diluted in 5 ml of ABB and centrifuged at 100,000 x g during
248 90 min. The MV pellet was resuspended in 500 µl of ABB and data were collected in a
249 flow cytometer (FACScalibur, BD Biosciences).

250

251 **MV staining**

252 MVs were stained with the lipophilic dye PKH-67 (Sigma Aldrich, USA) for capture
253 assays. 6 µl of PKH-67 was diluted in 1 ml of diluent C and MVs were also diluted 1/40
254 in diluent C. Both dilutions were mixed together at a volume ratio of 1:1, and labelling
255 was continued for 15 min at room temperature in the dark. The reaction was stopped by
256 adding 2 ml EV-free FBS (>16h at 100,000 x g), and MVs were then washed in PBS,
257 and ultracentrifuged at 100,000 x g for 1h 10min (SW28 rotor, OptimaTM XL-100K
258 Ultracentrifuge, Beckman).

259

260 **NanoSight analysis**

261 EVs were resuspended in 100 µL of PBS, 50 µL of which was diluted 1:10 with 450 µL
262 of PBS, and analyzed using NanoSight LM10 equipment and NTA software version 2.3
263 (NanoSight Ltd, Malvern, UK). Images were recorded for 60s (5 technical replicates)
264 with the following parameters: camera shutter - 1492, camera gain - 512, detection
265 threshold - 10.

266 **MVs capture assay**

267 To assess the ability of iDCs to capture MVs, 10⁵ iDCs were incubated at 37°C in 5%
268 CO₂ with PKH-67 labelled MVs (25 µg or 12.5 µg) at a final volume of 150 µl complete

269 medium. As a control, iDCs were incubated at 4°C. Several incubation times were
270 assessed in the different experiments.

271 After incubation, cells were extensively washed in cold PBS. At this point, they were
272 either stained for capture and phenotype analysis by flow cytometry or left in complete
273 medium at 37° C for a further 24 hours. After culture, cells were assessed for
274 expression of both activation markers and for allostimulation capabilities. For
275 phenotype analysis, the following murine mAbs were used (BD Biosciences): CD83-
276 APC, HLA-DR-APC-H7, CD25-PE and CD25-PE-Cy5. Isotype-matched mAbs were
277 used as controls. All analysis was performed in a FACS Canto II flow cytometer (BD
278 Biosciences) and analysed using FlowJo software. For inhibition experiments, cells
279 were treated for 30 min at 37°C with cytochalasin D (Calbiochem, Germany) at the
280 indicated concentrations prior to the addition of MVs.

281 **Allostimulation assay**

282 Allostimulation assays were performed by culturing together 1h-MV pulsed, 24h-
283 resting MDDCs with allogeneic CFSE-labelled T cells. T cells were isolated from
284 healthy donors' PBMCs by negative magnetic selection using the Easysep Human T
285 cell Enrichment Kit (Stemcell Technologies), and stained with CFSE (0.4 µM;
286 Invitrogen).

287 iDCs were co-cultured with T cells at different ratios, from 1:20 (5,000 MDDCs:
288 100,000 T cells) to 1:160 (625 MDDCs: 100,000 T cells). As a positive control, T cells
289 were stimulated with Phorbol 12-Myristate 13-Acetate (PMA, 0.6 ng/ml, Sigma
290 Aldrich) and Ionomycin calcium salt (200 ng/ml, Sigma Aldrich).

291 After 4.5 days of culture, proliferation was assessed by flow cytometry (LSR Fortessa
292 Analyzer, BD). Proliferative T cells were gated by diluted CFSE intensity.

293

294 **Cytotoxicity (viability) tests**

295 We used 5,000 Da (Wako Pure Chemical Industries Ltd., Osaka, Japan) or 500,000 Da
296 (Nacalai Tesque Inc., Kyoto, Japan). Cell viability assays were performed
297 according to our previously reported method. Cells were inoculated in 96-multiwell
298 plates (Costar, Corning, NY, USA) at a cell density of 1.2×10^5 cells/well. At
299 confluence, cells were incubated with serially diluted DSS for pre-determined time
300 periods. DSS was dissolved in culture media and filter-sterilized using a 0.45 µm filter.

301 Viability was assayed by a commercially available kit (Cell Titer 96™ AQueous,
302 Promega, Madison, USA), which depends on the physiologic reduction of MTS to
303 formazan. Analyses were performed in triplicate.

304

305 **Confocal microscopy assays.**

306 To corroborate MV capture and examine the distribution of MVs in pulsed iDCs, 10⁵
307 iDCs were incubated with PKH-67-labelled MVs from *Giardia intestinalis* at 37°C for
308 the indicated periods. Then, cells were extensively washed, stained for CD11c-PE
309 (ImmunoTools), and fixed with 2% formaldehyde solution. Fixed cells were mounted in
310 Immunofluorescence slides with ProLong® Gold Antifade Reagent with DAPI (Life
311 Technologies), and were examined in an Axio-Observer Z1 inverted fluorescent
312 Microscope (ZEISS, Germany).

313 To determine the endocytosis trafficking, MVs-pulsed iDCs were stained for the Early
314 Endosome Antigen (EEA-1; BD Transduction Laboratories) and Transferrin Receptor
315 (TfR; Abcam, UK), followed by Alexa546-anti mouse IgG and anti rabbit IgG,
316 respectively. Labelling was performed with the IntraStain fixative and permeabilization
317 kit (Dako, Denmark). Finally, cells were cytopun onto glass slides and mounted with
318 ProLong® Gold Antifade Reagent with DAPI. Confocal microscopy was performed on
319 an Axio-Observer Z1 microscope with the LSM 70 confocal module (ZEISS,
320 Germany).

321 **Proteomic assays.**

322 Samples were digested with sequencing grade trypsin (2.5 ng/μL; Promega) as
323 described elsewhere (Shevchenko, A et al, 1996). The digestion mixture was dried in a
324 vacuum centrifuge, resuspended in 50 μL of 2%ACN, 0.1% TFA 1 μl of each digested
325 mixture were loaded onto a trap column (NanoLC Column, 3μ C18- CL, 100umx15cm
326 ;Nikkyo), and desalted with 0.1% TFA at 2μl/min during 10 min. The peptides were
327 loaded onto an analytical column (LC Column, 3 μ C18- CL, 75umx12cm, Nikkyo)
328 equilibrated in 5% acetonitrile 0.1% FA (formic acid). Peptide elution was carried out
329 with a linear gradient of 5 to 35% buffer B in 120 min (A: 0.1% FA; B: ACN, 0.1% FA)
330 at a flow rate of 300nl/min. Peptides were analyzed in a mass spectrometer nanoESI
331 qQTOF (5600 TripleTOF, ABSCIEX). The tripleTOF was operated in
332 information- dependent acquisition mode, in which a 0.25- s TOF-MS scan from 350–

333 1250 m/z, was performed, followed by 0.05- s product ion scans from 100–1500 m/z on
334 the 50 most intense 2- 5 charged ions. The MS/MS information (combined from three
335 runs of one sample) was sent to MASCOT v2.3.02 or to PARAGON via the Protein
336 Pilotv 4.5 (ABSciex).

337 MASCOT search engine (Matrix- Science). Database search was performed on
338 NCBI *Giardia* EST. Searches were performed with tryptic specificity allowing one
339 missed cleavage and a tolerance on the mass measurement of 50 ppm in MS mode and
340 0.6 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification
341 and oxidation of Met, and deamidation of Asn and Gln as variable modifications.

342 ProteinPilot v4.5. search engine (ABSciex). ProteinPilot default parameters were used
343 to generate peak list directly from 5600 TripleTof .wiff files. The Paragon algorithm of
344 ProteinPilot was used to search the NCBI protein database with the following
345 parameters: trypsin specificity, cys- alkylation, no taxonomy restriction, and the search
346 effort set to through. To avoid using the same spectral evidence in more than one
347 protein, the identified proteins were grouped based on MS/MS spectra by the
348 Protein- Pilot Progroup algorithm. Thus, proteins sharing MS/MS spectra are grouped,
349 regardless of the peptide sequence assigned. The protein within each group that can
350 explain more spectral data with confidence is shown as the primary protein of the group.
351 Only the proteins of the group for which there is individual evidence (unique peptides
352 with enough confidence) are also listed, usually toward the end of the protein list.

353

354

355 **Results**

356 **1-Trophozoites from *Giardia intestinalis* release extracellular vesicles (MVs) from** 357 **the plasma membrane under different environmental conditions.**

358 To simulate the dramatic environmental changes during the cell cycle of *Giardia*
359 *intestinalis*, we compared the growth curve of trophozoites cultured *in vitro* for 48 h
360 under different pHs ranging from 5 to 8. A poor growth was obtained at pH 8.0, and the
361 best condition was at pH 7.0. Curiously, the parasite was able to grow at acidic pHs
362 (pH 5.0 and 6.0), and the growth was highly inhibited at pH 8.0., a condition in which
363 trophozoites are differentiated to cyst when the parasites are in the presence of inducers
364 such as bile, (Figure 1A). Due to these growth curve differences, we next analysed
365 whether the trophozoites could release MVs in response to different environmental
366 conditions (Figure 1B). We saw a high release of MVs at 24 h at pH 7.0, and no
367 differences were found at different pHs after 48 h (Figure 1C).

368 We also analyzed the production of MVs during the first 3 h in a serum-free medium
369 with added calcium, a well-known microvesicle inducer, and we detected an increase of
370 MV release between 30 to 120 min, maintaining the release up to 180 min (Figure 1D).
371 On this basis, we decided to perform an experiment of MV induction for 60 min only.

372 To characterize the type of MVs released by the trophozoite forms of *Giardia*
373 *intestinalis*, we analyzed the supernatant of trophozoites cultured in a fetal bovine
374 serum-free medium for 60 min in the presence of 1 mM of calcium, and subjected to
375 different rounds of centrifugation and final ultracentrifugation. Examination of the
376 preparation by electron microscopy revealed cup-shaped vesicles of 60–150 nm (Not shown) .
377 MVs were also quantified by FACS and analyzed for size and granularity and the
378 presence of phosphatidylserine by detecting annexin V-FITC staining (Supplementary
379 Fig. 1). We verified the impact of calcium on MV release using higher concentrations
380 of calcium, as well as EGTA (calcium chelating inhibitor), and a calcium ionophore, an
381 activator of calcium release, as shown in the figure 1E.

382 To determine vesicle size variation in the population of extracellular vesicles, we used
383 nanoparticle tracking analysis (Nanosight, Costa Mesa, CA) to directly examine millions of
384 vesicles. This analysis showed a peak with a mean diameter of 166 nm and that 80 % were
385 between 130 –150 nm in diameter (Fig. 1F and 1 G)

386

387

389 2- The origin of microvesicles is plasma membrane and lipid raft dependent.

390 Due to the size of MVs and presence of phosphatidylserine at the surface, we were
 391 interested to verify plasma membrane and lipid raft (also named DRMs (detergent-
 392 resistant membranes)) involvement in the biogenesis of *G. intestinalis* MVs. We
 393 investigated whether the disruption of lipid rafts from trophozoite plasma membranes
 394 affected MV formation, as it was defined previously (Ian del Conde et al, 2005). To
 395 assess this effect, we treated trophozoite forms with M β CD (methyl- β -cyclodextrin) at
 396 different concentrations, and we saw a dose-dependent inhibition of MV formation from
 397 25 to 60 % with increasing M β CD (2.5 μ M to 10 μ M), showing that the depletion of
 398 membrane cholesterol decreases EMV formation (Figure 2 A).

399 The inhibition of MV formation by removal of cholesterol suggests that trophozoites of
 400 *G. intestinalis* may diminish the parasite's ability to attach to host cell.???????WHY
 401 We next performed the adhesion assay using trophozoites either non-treated or treated
 402 with 2.5- 10 μ M of M β CD, and we detected a dose-dependent inhibition of the
 403 attachment of *G. intestinalis* to host cells (Figure 2B).

404 The importance of lipid raft structure and MV release in the attachment to the host cell
 405 was notably verified when *G. intestinalis* treated with 5 μ M of M β CD was used in an
 406 adhesion assay for 3 h in the presence of MVs. We saw that the parasites treated with 5
 407 μ M M β CD were unable to attach to Caco-2 cells, and the addition of MVs restored the
 408 ability to attach in a dose-dependent manner (Figure 2C) ?????where is this??. These
 409 results suggest that MVs bring back certain physical properties to the membrane of the
 410 trophozoites, and probably increase the presence of a putative molecule-receptor
 411 ligation.

412

413 3- Trophozoites from *Giardia intestinalis* secrete MVs that aid parasite attachment 414 to host cells.

415 To investigate whether MVs from *G. intestinalis* could facilitate the adhesion of
 416 trophozoites to host cells, we performed an adhesion assay using monolayers of Caco-2
 417 cells, that resemble the enterocytes lining the small intestine, and trophozoites of *G*
 418 *intestinalis* WB strain. Monolayers of Caco-2 cells in the semi-confluent state were
 419 incubated with 1×10^6 trophozoites of *G. intestinalis* in the presence of different
 420 concentrations of MVs from *G. intestinalis*, for 1 and 3 h at 37 °C and 5 % CO₂. Cells
 421 were then washed, stained with Giemsa and quantified by light microscopy. We
 422 detected a slight dose-dependent increase of *G. intestinalis* attachment to Caco-2 cells at

423 1 h, and a strong 3-fold increase of adhesion of *Gintestinalis* to intestinal cells when
424 parasites and host cells were incubated with 7 µg of MVs compared to that without
425 MVs. (Figure 3 A). Microvesicles treated with proteinase K ((0.05 mg/ml;
426 SigmaAldrich)for 1 hour at 37°C and heat inactivated for incubation at 95°C for 10
427 minutes were used as adhesion assays controls. The treated-MVs had no effect on the
428 attachment of trophozoites to Caco cells (not shown)

429 To analyze the specificity of this MV-mediated effect, we next used MVs of
430 *Gintestinalis* in an invasion assay, using metacyclic trypomastigotes forms of *T. cruzi*
431 and Vero cells. Even using higher concentration of MVs (10 µg), the rate of metacyclic
432 trypomastigotes invasion was not modified in the presence of *G intestinalis* MVs that
433 the Mvs could have a specific effect on the parasite and not on Vero Cells.(data not
434 shown). **Too much data not shown???**

435

436 4- MVs communicate with neighbouring cells and face innate immunity.

437 Having shown the importance of MVs for cell adhesion, we next investigated the
438 impact of MVs on neighbouring cells and in stimulating innate immunity. For this
439 purpose, we analysed the effect of MVs on the viability of Caco-2 cells and the ability
440 of MVs to be captured by and modulate DC function.

441 Firstly, Caco-2 cells were incubated in the presence of different concentrations of MVs
442 derived from *Gintestinalis* for 24 and 48 h, and a MTS viability assay was performed.
443 After three independent experiments, we did not detect any effect on the viability of
444 Caco-2 cells using different concentrations of MVs (Figure 3B). We then analysed the
445 interaction between MVs and DCs. Vesicles from GI were labelled with the aliphatic
446 fluorophore PKH-67, and the interaction between MVs and iDCs was assessed by
447 capture, phenotype of capturing cells, and alloproliferation analysis (Figure 4).

448 Time-course experiments revealed that *G. intestinalis* MVs are rapidly internalized by
449 iDCs (30-40% positively labelled cells only 30 minutes after pulse). Of note, capture of
450 MVs did not affect viability of iDCs (Figure 4A, 4B). Noticeably, capture was inhibited
451 almost completely at low temperature (4°C) and by the addition of cytochalasin D to the
452 iDC culture, thus indicating a clear endocytic component on the capture of MVs (Figure
453 4BandC). MV internalization was confirmed also by inverted fluorescent microscopy,
454 and cells pulsed at 4°C showed no MVs within them (Figure 4D). To further confirm the
455 involvement of the endocytic pathway in the MV capture by iDCs, confocal images
456 were taken at short time intervals. Five minutes after incubation, captured MVs

457 colocalized with the early endocytosis marker Transferrin Receptor (TfR) (Figure 4E).
458 At later time points (15 min of incubation), MVs colocalized with the Early Endosome
459 Associated protein (EEA-1, Figure 4E), and after one hour of incubation, cells showed
460 sac-like compartments where captured MVs probably aggregated all together (Figure
461 4D).

462 To assess the capacity of MVs to induce DC maturation, iDCs were pulsed for one hour
463 with MVs, washed, and further cultured for 24 h. At this time point, classical activation
464 markers such as CD83 and HLA-DR did not vary their expression. However, GI-MVs-
465 capturing iDCs upregulated the activation marker CD25 (Figure 5A) suggesting a mild
466 activation of iDCs. This mild activation was further confirmed by the increase of
467 alloantigenic stimulation potential of DCs in T cell alloproliferation experiments
468 (Figure 5B).

469

470 **5- Proteomic and RNA analysis of MVs suggest a modulation effect to host cells.**

471 We investigated the content of MVs released by mid-log trophozoites and by
472 trophozoites induced to Cyst form by proteomic assays. The mass spectrometry analysis
473 showed differences in the content of these MVs (Table 1). Only 11 proteins were
474 identified from the trophozoite stage, seven of them previously reported as present in
475 MVs in other organisms as described in the vesiclepedia (Kalra et al., 2012;
476 <http://microvesicles.org>). In the case of MVs from cyst transition, 80 proteins were
477 detected (36 of them still unknown), and from the 44 remaining, 24 proteins
478 corresponded to previously identified EVs proteins (Table 1). Interestingly, in the cyst
479 stage, proteins involved in pathogenesis like VSPs and giardins were identified in MVs
480 (Table 1). Further investigation is needed to identify specific markers released by the
481 different MV populations.

482 When analysing the RNA content of MVs released by trophozoites, there was a
483 presence of miRNA produced by calcium induction in trophozoites forms (Figure S1).
484 Interestingly, when we compared the total RNA from non-induced trophozoites with
485 trophozoites activated to produce MVs, a clear difference was noticed, where the
486 miRNA in trophozoites producing MVs contained the miRNAs that appear in MVs
487 purified after induction. (Fig S1).

488

489

490 Discussion

491

492 1- Trophozoites from *Giardia intestinalis* respond to environmental changes 493 releasing MVs that facilitate the interaction with host cells.

494 The results presented in this study have demonstrated that trophozoites from *Giardia*
495 *intestinalis* are able to release MVs in response to different pH levels, and calcium
496 (Figure 1). Many reports have shown the secretion of MVs during an interaction of
497 protozoans with host cells. One interesting example of the role of MVs affecting the
498 environment is the murine malaria model infected with *Plasmodium berghei*, which
499 develops cerebral malaria (CM). EVs isolated from plasma derived from infected
500 erythrocytes resulted in a potent activation of macrophages via toll-like receptors,
501 whereas plasma-derived MVs from naïve animals did not induce macrophages (Couper
502 et al., 2010). In the same murine model, the abrogation of MVs formation in mice
503 knocked out for the gene *ABCA1* protected these animals against CM, demonstrating a
504 link between EV production and pathogenesis (Combes et al., 2005).

505 In *Trypanosoma cruzi*, Cestari et al. (2012), demonstrated that metacyclic
506 trypomastigotes forms when in contact with the monocytic THP-1 cell line, release
507 MVs that inhibit C3 convertase, and aid the parasite to invade host cells. Recently, other
508 groups have shown that MVs from *T. cruzi* have different sizes, and different effects.
509 Bayer-Santos et al. (2013), found two types of EVs secreted by the parasites. Later,
510 Garcia-Silva et al. (2014); Linhares-Lacerda et al. (2015), and Fernandez-Calero et al.
511 (2015) showed the involvement of miRNA in MV transfer to host cells, indicating this
512 mechanism as a novel modulating effect against neighbouring cells.

513 Other reports have also described that the protozoan *Trichomonas vaginalis* secretes
514 extracellular vesicles similar to mammalian exosomes. The parasite-derived exosomes
515 contain RNA, conserved exosomal proteins and parasite-specific proteins (Twu et al.,
516 2013). They also demonstrated that *T. vaginalis* exosomes are able to deliver their
517 contents to host cells and modulate host cell immune responses. Interestingly, exosomes
518 from highly adherent parasite strains increased the adherence of poorly adherent
519 parasites to vaginal and prostate epithelial cells. In contrast, exosomes from poorly
520 adherent strains had no measurable effect on parasite adherence (Twu et al., 2013). We

521 believe that *Giardia intestinalis* release MVs as a response to differences in the
522 environment, and this is a likely consequence of limited organelle specialization in this
523 protozoan (Faso and Hell, 2011).

524

525 **2- Microvesicle release is associated with lipid rafts and MVs are derived from the**
526 **plasma membrane.**

527 The mechanism of MV release has been associated with induction or stimulation of
528 cells producing an increase in intracellular calcium, which is mobilized by calpain in
529 turn inhibiting flippase and activating scramblase. The biophysical step including the
530 exovagination of the phospholipid bilayer is a mechanism still poorly understood.
531 Different reports have shown that cholesterol should influence the lipid raft
532 microdomains on the membrane that participate in MV formation. An interesting report
533 by del Conde et al. (2005), demonstrated that the coagulation process is dependent of
534 the transference of tissue factors released from monocyte-MVs to platelets. This release
535 is dependent of lipids raft microdomains. Moreover, the shedding of MVs containing
536 tissue-factor was abolished with the depletion of membrane cholesterol. Interestingly
537 the giardia genome (Giardia DB) has no annotation to GPI-anchor proteins, a family of
538 proteins associated to sphingolipids and cholesterol on the plasma membrane and
539 related to lipid raft formation. However a putative transamidase annotated could be
540 transferring GPI to other acceptors. The recently described genome in *G. intestinalis*
541 (Morrison et al, 2007) revealed the presence of lipids synthesis and metabolic genes.
542 Which proteins and structures are related to lipids rafts formation need to be validated.
543 Recently De Chaterjee et al. (2015) hypothesized that Lipids rafts act as molecular
544 sensors on the plasma membrane. They used conjugated cholera toxin B (CTXB)
545 which binds GMI glycolipid demonstrating lipid rafts at the membrane, ventral disc and
546 caudal flagella. Moreover nystatin and filipin III, two well known LR disrupting agents
547 inhibited the CTXB binding indicating that lipid rafts contain cholesterol and the
548 removal destabilized the microdomains. De Chaterjee et al. (2015) discussed the
549 possible connection between lipids rafts and sphingolipids metabolism in *Giardia*
550 regulating the encystation process.

551

552 Our findings support the involvement of cholesterol in MV release, as we observed an
553 inhibition of MV production using different concentrations of M β CD (Figure 2A).
554 Furthermore, the absence of cholesterol inhibited the parasites attachment to the host
555 cell, and it was subsequently restored by the exogenous presence of MVs (Figure 2B).

556

557 **3-Microvesicles from *Giardia intestinalis* modulate dendritic cells implicating them** 558 **in the pathogenesis**

559 Factors associated with the pathology of giardiasis indicate a likely mechanical
560 alteration of intestinal mucosa for the attachment of the trophozoites, due to the
561 involvement of cysteine protease activities secreted by the parasite in contact with host
562 cells. In fact, a lower activation or damage to host cell should be associated with a low
563 inflammation that progresses in giardiasis. Previous work using mouse models to
564 determine the role of various cytokines in immunity to *Giardia* have shown that IL-6
565 plays a critical role in the control of primary infections with this parasite (Bienz et al.,
566 2003; Zhou et al., 2003). Here, we have analysed whether MVs release by trophozoites
567 should alter dendritic cells, which are a family of professional antigen-presenting cells
568 (APCs), that reside in all peripheral tissues in an immature state, capable of antigen
569 uptake and processing. Dendritic cells (DC) when activated are key to enhanced
570 cytokine secretion and enable DC migration and recruitment of other cell types. DC
571 capture of *Giardia* MVs suggests an effective contact with host cells. Furthermore, *in*
572 *vitro* or *in vivo* assays could indicate the role of MVs in dendritic cell modulation.
573 Recently, some manuscripts have shown and reviewed the role of dendritic cells during
574 protozoan-host cell interaction (i.e. Weidner et al., 2016; Feijo et al., 2016; Ersshing et
575 al., 2016). However, there are no current reports involving MVs from parasites in
576 modulating dendritic cell functions.

577

578 **4-Exosome and microvesicles content can alter neighbouring cells.**

579 There are numerous examples in the literature of protozoa releasing large amounts of
580 material into the extracellular space as a form of cellular communication with host cells
581 (Garcia-Silva et al., 2014; Linhares-Lacerda et al., 2015; Fernandez-Calero et al., 2015;
582 Cestari et al., 2012). In *Giardia intestinalis*, the diarrhoea and malabsorption could be a
583 direct result of the interaction of the parasite with the intestinal epithelium which might

584 be mediated by the parasite itself, or by substances it secretes or by MVs that could alter
585 neighbouring cells. Analysis of Caco-2 human intestinal epithelial cells demonstrated
586 that *Giardia* infection resulted in a strong alteration of the expression profile of these
587 cells, including stress-response genes and chemokines, as seen by microarray analyses
588 (Roxstrom-Lindquist et al., 2005). Transcriptional changes in *G. intestinalis* during
589 interaction with intestinal epithelial cells were also monitored by microarray analysis of
590 *G. intestinalis* cDNAs, and indicated up-regulation of genes encoding enolase, cysteine
591 proteinase, arginine deiminase and oxygen defence proteins (Ringqvist et al., 2011).
592 Interestingly, contact of *G. intestinalis* with epithelial cells resulted in the release of
593 metabolic enzymes (arginine deaminase, ornithine carbamoyltransferase and enolase)
594 from *G. intestinalis*, which disabled host immune factors including nitric oxide
595 (Ringqvist et al., 2008). A previous study also demonstrated that the excretory-secretory
596 products (ESP) of *G. lamblia* contained major antigen(s) responsible for protection
597 against infection in mice (Kaur H et al, 1999). Oral administration of *G. lamblia* ESPs
598 into BALB/ c mice stimulated a Th2 response, which led to intestinal histological
599 changes characterized by eosinophilic infiltration, and can induce host cell apoptosis,
600 hypercellularity, and enterocytic desquamation (Jimenez et al., 2004). Glycoproteins in
601 *G. lamblia* ESP were found to induce antibody production during giardiasis (Khar et al.,
602 2001; Jimenez et al., 2007). Cysteine proteases present in *G. lamblia* ESP were essential
603 for the induction of antibody and cytokine production in BALB/ c mice infected with
604 ESP (Jimenez et al., 2009). Our findings of small RNAs in the cargo of EMVs suggest
605 that the parasite could transfer material to host cells through EMVs, modifying the cell
606 phenotype. An interesting previous report supports this possibility; the infection of
607 human ileocecal adenocarcinoma cell line HCT-8 with *Giardia intestinalis* can induce
608 host cell apoptosis. Signs of chromatin condensation and caspase 3 activation was found
609 to occur in monolayers exposed to different *G. intestinalis* assemblages (Kho et al.,
610 2013). Nuclear fragmentation and cell death was suppressed with a caspase 3 inhibitor.
611 The most important point was the demonstration that cellular extracts from *Giardia*
612 *intestinalis* were able to induce apoptosis without contact with the parasite. This fact
613 supports the idea that EVs should alter the host cell. MicroRNAs have been shown to
614 have a role in cancer, cell re-programming, hypertension regulation and other chronic
615 diseases (reviewed by Tao ZQ et al., 2016; Stepicheva and Song, 2016; Makarova et
616 al., 2016), and could be essential in host-parasite cell interactions.

617 Taken together, our findings and new concepts reveal another facet to the ever
618 increasingly complex environment of dynamic cellular communication between
619 parasites and hosts. The content of MVs, including protein and nucleic acids, may
620 modify the cell phenotype avoiding innate immunity and producing the infection
621 (Figure 6). The identification of cellular targets and inhibitors of microvesiculation
622 could potentially represents a novel strategy to control the diseases, and are currently
623 under investigation.

624

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626

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634

635

636

637

638 **FIGURE LEGENDS**

639 **FIGURE 1. Stress factors in the gastric environment affect growth of *G.***
640 ***intestinalis* trophozoites and their production of MVs:** A) Growth at different pH
641 values. An initial inoculum containing 5×10^4 parasites/ml was cultured at 37°C for 24 or
642 48 h in media at different pH values. Subsequently, parasites were quantified using a
643 Neubauer chamber. Results are representative of two independent experiments. B) MV
644 production with different inducers. Trophozoites were induced to release MVs in
645 different conditions, the supernatant then being ultracentrifuged, and MV production
646 quantified by flow cytometry. The chart is representative of measurements made in
647 triplicate, and the results represent the mean \pm standard error of three independent
648 experiments. C) Effect of pH on MV production by *G. intestinalis* trophozoites. MVs
649 were induced in the presence of CaCl_2 and their release was monitored at 24 and 48h
650 time points. EMVs contained in the supernatant were quantified by flow cytometry. The
651 results are representative of measurements made in triplicate \pm standard error of three
652 independent experiments. D) Early kinetics of MV production by trophozoites of *G.*
653 *intestinalis* at pH 7.0 MVs induction was performed with CaCl_2 at pH 7 from 0 to 180
654 min, and the analysis of the release was performed after different incubation times. MVs
655 contained in the supernatant were quantified by flow cytometry. The chart is
656 representative of measurements made in triplicate, and the results represent the mean \pm
657 standard error of three independent experiments. E) MV production from *G. intestinalis*
658 trophozoites upon treatment with 1 mM CaCl_2 , 3 μM Calcium ionophore A23187 and 5
659 mM EGTA. F) Nanosight trace of purified vesicles. A mean diameter of nm
660 was measured. (G) Table from Nanosight analysis of percentage of purified vesicles in various
661 size ranges.

662

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664

665

666 The bar graph represents the mean \pm standard error of three independent experiments.

667 **Figure 2 Effect of lipid raft disruption by cholesterol depletion on MV production.**

668 A) The extraction of membrane cholesterol decreases the production of MVs.
669 Trophozoites were incubated with different concentrations of M β CD for one hour. Later
670 the trophozoites were induced with 1 mM of CaCl_2 , and the MVs released were
671 quantified by flow cytometry. Measurements were made in triplicate and the results

672 represent the mean \pm standard error of three independent experiments. The Student t test
673 was used for statistical analysis. The asterisks WHERE?? indicate the results that were
674 statistically significant ($p < 0.05$) compared to control. B) Treatment of *Giardia*
675 *intestinalis* WB with the lipid raft-destabilizing methyl- β -cyclodextrin (M β CD) inhibits
676 their adhesion to Caco 2 cells. Adhesion of WB pre-treated with 0- 10 μ M of M β CD
677 to Caco cells was determined for 3 hrs at 37°C. The trophozoites present in the
678 supernatant were quantified by hemocytometer. C) MVs of *Giardia intestinalis* restore
679 the ability of GI WB pre-treated with 5 μ M of M β CD to bind to Caco cells. The
680 Adhesion of WB pre-treated with 5 μ M of M β CD to Caco cells was determined in
681 the presence of different concentrations of purified MVs of GI for 3 hrs at 37 C.
682 Results represent the mean \pm the standard error from two independent experiments. The
683 Student t test was used for statistical analysis. The asterisks indicate the results that
684 were statistically significant ($p < 0.05$) compared to control.

685

686 **Figure 3 – Effect of *Giardia intestinalis* microvesicles on parasite adherence to**
687 **Caco-2 cells.**

688 A) Trophozoites adherence to host cells in the presence of MVs. Trophozoites were
689 incubated for 1 h ?? and 3 h ??, with Caco-2 cells in the presence of different
690 concentrations of MVs (previously obtained from trophozoites). The amount of
691 adhering trophozoites to Caco-2 cells was determined by counting the trophozoites
692 present at the supernatant in a Neubauer chamber. The conditions were tested in
693 duplicate. As control Isolated vesicles were incubated with proteinase K (0.05 mg/ml;
694 SigmaAldrich) for 10 minutes at 37 C. The Results represent the mean \pm standard error
695 of three independent experiments. The Student t test was used for statistical analysis.
696 The asterisks where indicate the results that were statistically significant ($p < 0.05$)
697 compared to control. B) Effect of *G. intestinalis* MVs on viability of Caco-2 cells,
698 5×10^3 cells/well being incubated in the presence of different concentrations of MVs
699 from *G. intestinalis* trophozoites for 24 h. Cell viability was assessed by the MTS assay
700 and the results are presented as percentages relative to untreated cells with MVs
701 (control). Measurements were made in triplicate, the results represent the mean \pm
702 standard error of three independent experiments. ANOVA with multiple comparisons

703 was performed the statistical analysis. The asterisks indicate the results that were
704 statistically significant ($p < 0.05$) respect to the control.
705

706 **Figure 4. MVs from *Giardia intestinalis* are actively captured by human immature**
707 **dendritic cells (iDCs).** A) and B) MVs labelled with PKH-67 were added to iDCs and
708 capture was assessed after 30minutes or 1h at 37°C or 4°C without or with further 24h
709 of resting. The percentage of PKH-67⁺ iDCs was analysed by flow cytometry. A)
710 Representative plots of the capture analysis performed by flow cytometry. B) Mean
711 values of PKH67⁺ cells \pm SD of three independent experiments. C) Active capture by
712 iDCs was corroborated by blocking with Cytochalasin-D incubation 30min prior to
713 EMV addition. The mean \pm SD of the viability (%7AAD⁻ cells) and capturing cells
714 (%PKH-67⁺ cells) is expressed as relative to the control. D) Inverted fluorescence
715 imaging of iDCs pulsed with PKH-67- labelled MVs confirmed temperature-
716 dependence of EMV internalization by iDCs (CD11c⁺, red) (b). After one hour of
717 incubation, iDCs aggregate endocytosed MVs in sac-like compartments (c and d). E)
718 MVs are internalized and sorted to the endocytic compartments of iDCs. Confocal
719 imaging of iDCs pulsed with PKH-67- labelled MVs show co-localization (yellow) with
720 transferrin receptor (TfR, red; a and b), at already 5 min after pulse. Within 15 min,
721 MVs co-localized with the early endocytosis associated protein (EEA-1, red, c and d),
722 showing aggregation of MVs in endosomal compartments.

723 **Figure 5. MVs from *Giardia intestinalis* induce activation and enhance the**
724 **allostimulation capabilities of iDCs.** MVs were added to iDCs and cultured for 1h.
725 After extensive washing and 24h resting, cells were checked for (A) activation marker
726 expression (CD83, HLA-DR and CD25) and (B) allostimulation capabilities towards T
727 cells. A) Fold change expression of the indicated markers, relative to non-pulsed iDCs.
728 Data is depicted as mean \pm SD of three independent experiments. B) CFSE-labeled T
729 cells were co-cultured with 1h-pulsed, 24h-resting iDCs at the indicated ratios.
730 Alloproliferation of T cells was analysed according to the percentage of CFSE^{low} cells.
731 Data is representative of three independent experiments.

732 **Figure 6. *G. intestinalis* MVs increase parasite adherence to intestinal epithelial**
733 **cells but uptake by iDC cells may through their activation help direct a T cell**
734 **response.** (A) *G. intestinalis* trophozoite EMVs increase the adherence of the parasite to

735 intestinal epithelial cells. (B) *G. intestinalis* MVs are taken up by iDCs which are then
736 activated and mature (showing upregulation of CD25). T cell alloproliferation
737 experiments then showed an increase of the alloantigenic stimulation potential of DCs.

738

739 **TABLE 1. Proteomic analysis of microvesicles from *Giardia intestinalis* in**
740 **trophozoites and cyst stages.** Genbank accession number, name of the gene, relative
741 abundance by EmPAI data, and presence in homologues dataset of MVs
742 (microvesicles.org) are shown.

743

744 **Supplementary Figure 1.**

745 **Flow cytometric analysis of the MVs of *G.intestinalis*.** The MVs were quantified by
746 flow cytometry (BD FACScalibur) in size and granularity graphs (FSC x SSC). PS
747 exposure analysis was performed with FITC conjugated Annexin V on a FL-1
748 fluorescence histogram. (A) FSCxSSC plot of MVs labeled with annexin-V-FITC, the
749 population of MVs with surface PS exposure (green, R2) were obtained from a
750 fluorescence histogram (B), as marking control were used MVs without Annexin-V-
751 FITC; (C) 1 mM CaCl₂; (D) 3μM Calcium ionophore A23187; (E) 5mM EGTA;

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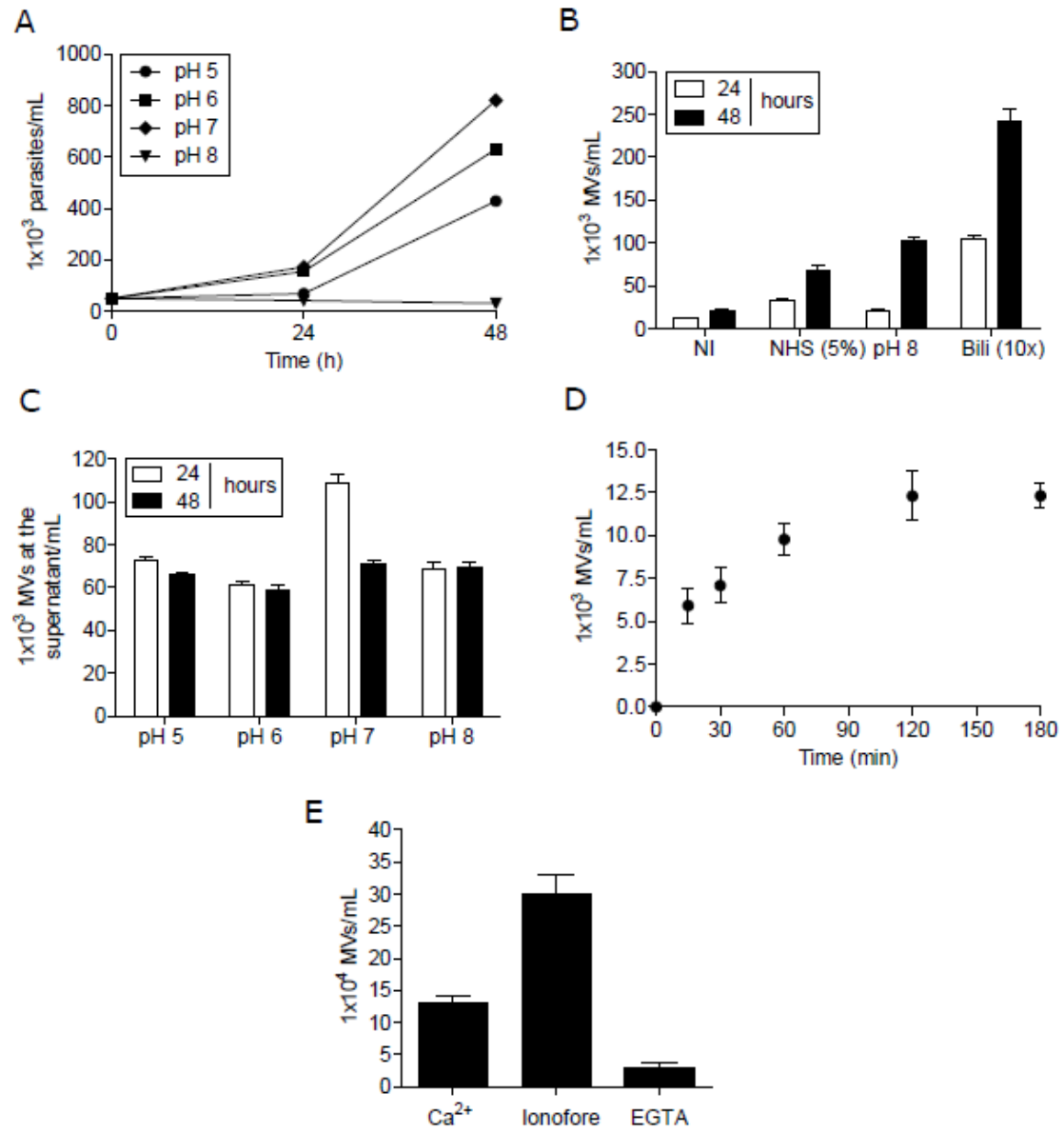


Fig. 1

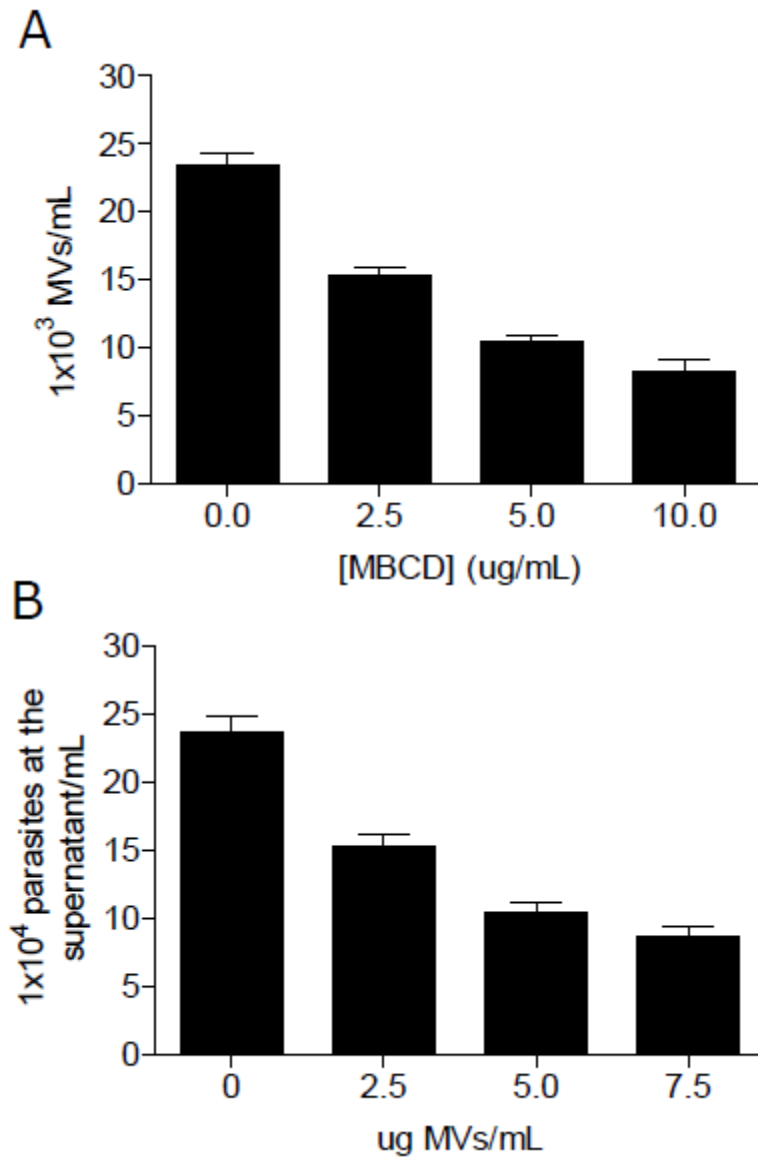


Fig. 2

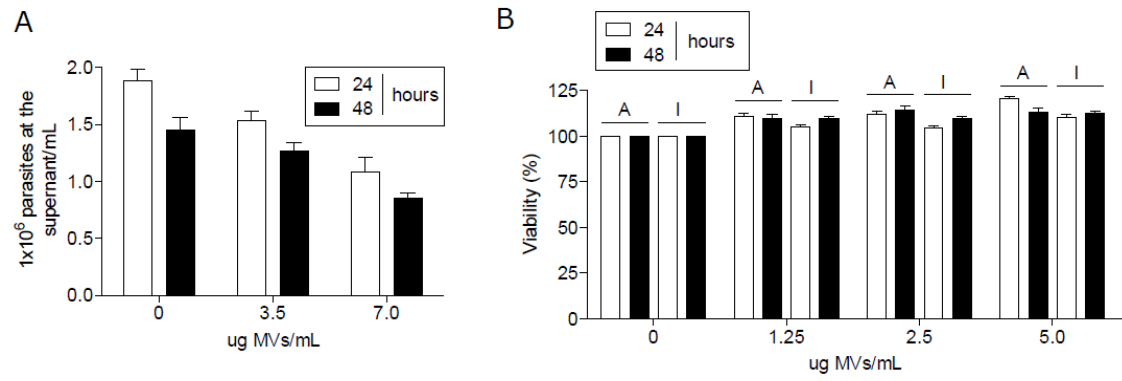
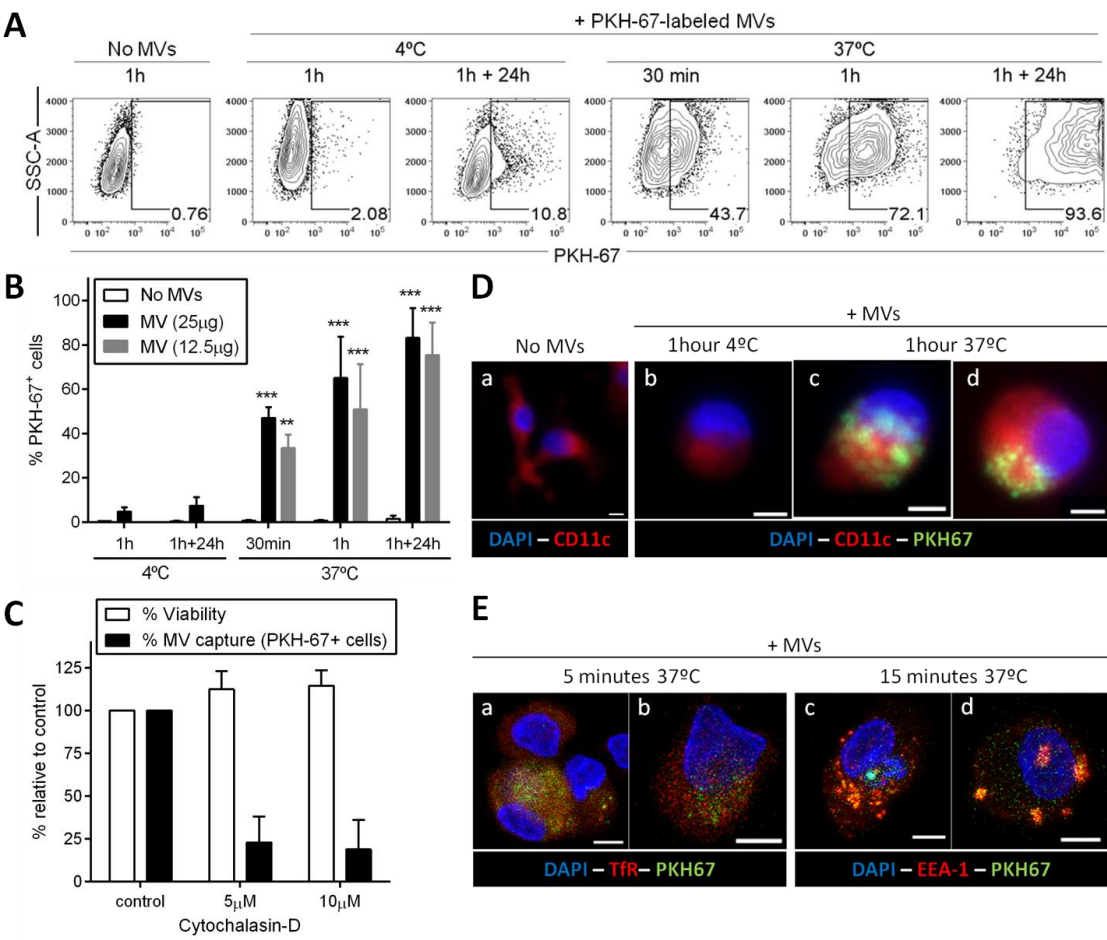


Fig. 3



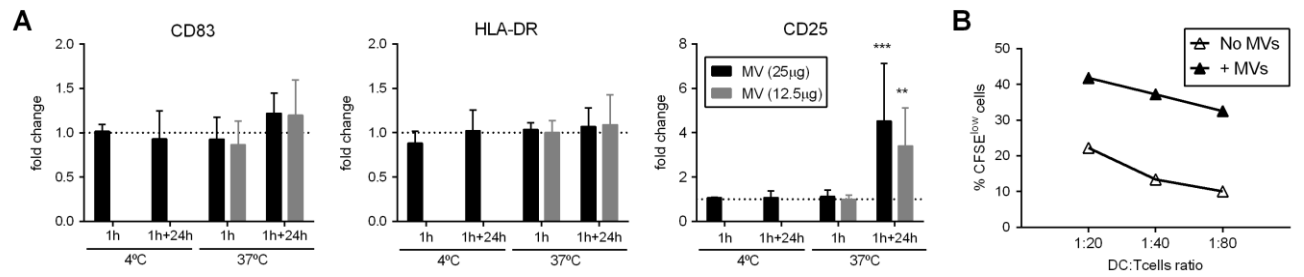
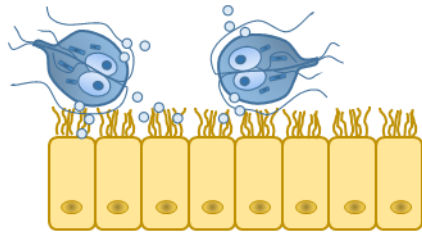


Fig. 5

A



B

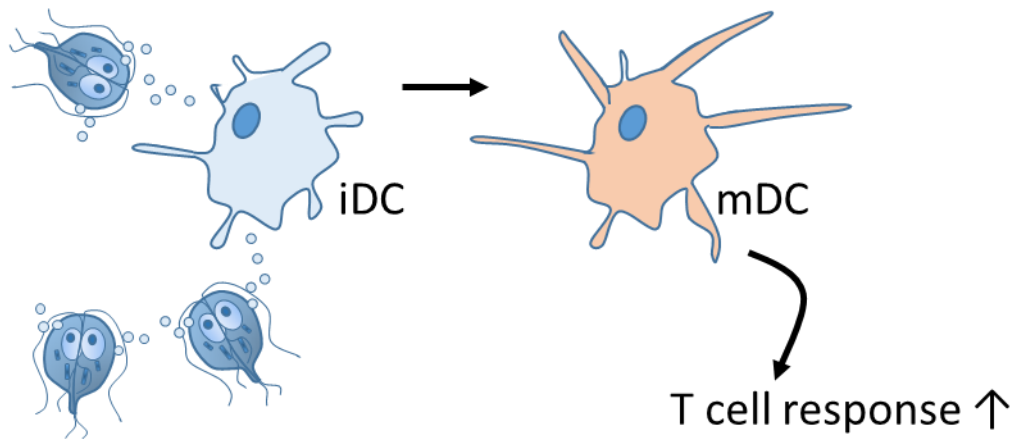


Fig. 6

Table 1- Proteomics analysis of different kind of *Giardia intestinalis* Microvesicles

Accession	Name	Total EmpAI	Homologues EVs
1.2 MVs TROPHOZOITE			
tr A8BPC0 A8BPC0_GIAIC	Alpha-tubulin	2.79	yes
tr E2RTT6 E2RTT6_GIAIC	Ornithine carbamoyltransferase	2.42	yes
tr A8BSP4 A8BSP4_GIAIC	Glyceraldehyde-3-phosphate dehydrogenase	2	yes
tr E2RU36 E2RU36_GIAIC	Arginine deiminase	2	yes
tr E2RTN3 E2RTN3_GIAIC	Glucosamine-6-phosphate isomerase	2	yes
RRRRRtr A8BTS5 A8BTS5_GIAIC	Splicing factor-like protein, putative	1.04	no
RRRRRtr A8BUK9 A8BUK9_GIAIC	Dynein heavy chain, putative	0.75	yes
tr A8BS83 A8BS83_GIAIC	rRNA biogenesis protein RRP5	0.14	yes
RRRRRtr A8BBV7 A8BBV7_GIAIC	Uncharacterized protein	0.08	ND
tr A8BG55 A8BG55_GIAIC	Uncharacterized protein	0.06	ND
tr A8BAB7 A8BAB7_GIAIC	Uncharacterized protein	0.05	ND
2.2. MVs CYST 24H			
Cytoskeleton			
tr A8BEI6 A8BEI6_GIAIC	Beta tubulin	4.86	yes
tr A8BV70 A8BV70_GIAIC	Actin related protein	2.13	yes
tr E2RTW1 E2RTW1_GIAIC	Alpha-7.2 giardin	1.44	no
tr E2RTU4 E2RTU4_GIAIC	Alpha-11 giardin	0.43	no
tr A8BPC0 A8BPC0_GIAIC	Alpha-tubulin	0.11	yes
tr A8BER9 A8BER9_GIAIC	Median body protein	0.06	no
Membrane and transport			
tr A8BN35 A8BN35_GIAIC	Kinesin-like protein	0.14	yes
RRRRRtr A8BT70 A8BT70_GIAIC	Ciliary dynein heavy chain 11	0.11	yes
RRRRRtr A8B7D3 A8B7D3_GIAIC	Dynein heavy chain	0.11	yes
tr E2RU51 E2RU51_GIAIC	TMP52	0.15	no
RRRRRtr A8BGS8 A8BGS8_GIAIC	WD-repeat membrane protein	0.11	no
tr A8BUL9 A8BUL9_GIAIC	Cation-transporting ATPase 2, putative	0.06	yes
tr A8BK9 A8BK9_GIAIC	Phospholipid-transporting ATPase 1A, putative	0.06	yes
Metabolic enzymes			
tr E2RTT6 E2RTT6_GIAIC	Ornithine carbamoyltransferase	1.78	yes
RRRRRtr A8BQ11 A8BQ11_GIAIC	Phosphatidylinositol transfer protein alpha isoform	0.21	yes
RRRRRtr A8B3P9 A8B3P9_GIAIC	Acyl-CoA synthetase	0.02	yes
RRRRRtr A8B3D3 A8B3D3_GIAIC	Inorganic polyphosphate/ATP-NAD kinase, putative	0.08	no
RRRRRtr A8BKZ7 A8BKZ7_GIAIC	Protein tyrosine phosphatase-like protein	0.08	yes
tr A8BYC4 A8BYC4_GIAIC	Peroxisomal protein	0.28	yes
tr A8BVT2 A8BVT2_GIAIC	Midasin ATPase nuclear	0.12	yes
tr A8BW47 A8BW47_GIAIC	Kinase, NEK	0.1	no
RRRRRtr A8B8T1 A8B8T1_GIAIC	CDC19 Pyruvate kinase	0.08	yes
Nuclear proteins			
tr A8BUJ9 A8BUJ9_GIAIC	Histone H4	0.85	yes
RRRRRtr A8BYH2 A8BYH2_GIAIC	Histone H3	0.06	yes
RRRRRtr A8BTS5 A8BTS5_GIAIC	Splicing factor-like protein, putative	0.23	no
RRRRRtr E2RU53 E2RU53_GIAIC	Mre11 endonuclease	0.16	yes
tr A8B9T6 A8B9T6_GIAIC	Nuclear LIM interactor-interacting factor 1	0.11	no
RRRRRtr E2RU87 E2RU87_GIAIC	Mlh2-like protein	0.09	no
tr A8BN96 A8BN96_GIAIC	Reverse transcriptase/endonuclease, putative	0.06	yes
Surface proteins			
tr A8B1Y1 A8B1Y1_GIAIC	VSP	0.8	no
tr A8BZM3 A8BZM3_GIAIC	VSP with INR	0.39	no
tr A8B2E6 A8B2E6_GIAIC	VSP	0.3	no
tr A8BD73 A8BD73_GIAIC	VSP	0.24	no
Chaperones			
tr E2RU97 E2RU97_GIAIC	14-3-3 protein	0.56	yes
tr A8BCR6 A8BCR6_GIAIC	Cytosolic HSP70	0.53	yes
tr A8BX22 A8BX22_GIAIC	Stress-induced-phosphoprotein 1	0.07	yes
Other			
RRRRRtr A8BK23 A8BK23_GIAIC	Coiled-coil protein	0.17	no
tr A8B5R5 A8B5R5_GIAIC	Protein 21.1	0.23	no
RRRRRtr A8B463 A8B463_GIAIC	Protein 21.1	0.14	no
tr A8BAF5 A8BAF5_GIAIC	Coiled-coil protein	0.08	no
RRRRRtr A8BYJ8 A8BYJ8_GIAIC	Mucin-like protein	0.14	yes
tr A8B4Q1 A8B4Q1_GIAIC	NOD3 protein, putative	0.1	no
tr A8B4S4 A8B4S4_GIAIC	Retinoic acid induced 17-like protein	0.11	no
tr A8BNT5 A8BNT5_GIAIC	Ribosomal protein L9	0.12	yes
RRRRRtr A8BUV6 A8BUV6_GIAIC	Zinc finger domain protein	0.08	no
Unknown			
RRRRRtr A8BC17 A8BC17_GIAIC	Uncharacterized protein	0.32	
RRRRRtr A8B9Q6 A8B9Q6_GIAIC	Uncharacterized protein	0.22	
tr A8BHN6 A8BHN6_GIAIC	Uncharacterized protein	0.21	
tr A8BQ51 A8BQ51_GIAIC	Uncharacterized protein	0.21	
tr A8B4W6 A8B4W6_GIAIC	Uncharacterized protein	0.2	
tr A8BB64 A8BB64_GIAIC	Uncharacterized protein	0.19	
tr A8BSP2 A8BSP2_GIAIC	Uncharacterized protein	0.18	
tr D3KGH8 D3KGH8_GIAIC	Uncharacterized protein	0.15	
RRRRRtr A8B639 A8B639_GIAIC	Uncharacterized protein	0.15	
tr A8BFN9 A8BFN9_GIAIC	Uncharacterized protein	0.13	
tr A8B6D8 A8B6D8_GIAIC	Uncharacterized protein	0.12	
tr D3KI60 D3KI60_GIAIC	Uncharacterized protein	0.11	
tr A8BMW0 A8BMW0_GIAIC	Uncharacterized protein	0.1	
RRRRRtr A8BRV4 A8BRV4_GIAIC	Uncharacterized protein	0.1	
RRRRRtr A8BZ05 A8BZ05_GIAIC	Uncharacterized protein	0.1	
tr A8BAB7 A8BAB7_GIAIC	Uncharacterized protein	0.1	
tr A8B688 A8B688_GIAIC	Uncharacterized protein	0.1	

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